

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE FORM PTO-1390 (REV 10-2000)		ATTORNEY'S DOCKET NUMBER <b>P/2107-162</b>
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>		
INTERNATIONAL APPLICATION NO. <b>PCT/EP99/05890</b>	INTERNATIONAL FILING DATE <b>11 August 1999</b>	PRIORITY DATE CLAIMED <b>12 August 1998</b>
TITLE OF INVENTION <b>TRANSGENIC PLANTS AND PLANT CELLS WITH REDUCED EXPRESSION OF INVERTASE INHIBITORS</b>		
APPLICANT(S) FOR DO/EO/US <b>Thomas RAUSCH</b>		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).</p> <p>4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <ol style="list-style-type: none"> <li><input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</li> <li><input checked="" type="checkbox"/> has been communicated by the International Bureau.</li> <li><input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> <p>6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <ol style="list-style-type: none"> <li><input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</li> <li><input type="checkbox"/> have been communicated by the International Bureau.</li> <li><input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li><input type="checkbox"/> have not been made and will not be made.</li> </ol> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). - <b>unsigned</b></p> <p>10. <input checked="" type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). <b>Claims 1-21</b></p>		
Items 11 to 16 below concern document(s) or information included:		
<p>11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.</p> <p><input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p>		
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<p><b>Dorothy Jenkins</b> Name of Person Mailing Correspondence</p> <p><i>Dorothy Jenkins</i> Signature</p> <p><b>February 12, 2001</b> Date of Signature</p>		

U.S. APPLICATION NO. if known, see 37 CFR 1.4		INTERNATIONAL APPLICATION NO. PCT/EP99/05890	ATTORNEY'S DOCKET NUMBER P/2107-162
<b>09762782</b>		<b>CALCULATIONS PTO USE ONLY</b>	
17. <input checked="" type="checkbox"/> The following fees are submitted:			
<b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5) ) :</b>			
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1000.00			
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00			
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00			
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00			
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<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>		<b>\$ 860.00</b>	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		\$	
<b>CLAIMS</b>		<b>NUMBER FILED</b>	<b>NUMBER EXTRA</b>
Total claims	21	- 20 =	1 X \$18.00
Independent claims	3	- 3 =	0 X \$80.00
<b>MULTIPLE DEPENDENT CLAIM(S) (if applicable)</b>		- \$270.00	
<b>TOTAL OF ABOVE CALCULATIONS =</b>		<b>\$ 878.00</b>	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.		\$	
<b>SUBTOTAL =</b>		<b>\$ 439.00</b>	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).		\$	
<b>TOTAL NATIONAL FEE =</b>		<b>\$ 439.00</b>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property		\$	
<b>TOTAL FEES ENCLOSED =</b>		<b>\$ 439.00</b>	
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a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>439.</u> to cover the above fees is enclosed.		<b>Check No.</b> <u>3328</u>	
b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.			
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>15-0700</u> . A duplicate copy of this sheet is enclosed.			
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.			
SEND ALL CORRESPONDENCE TO: <b>OSTROLENK, FABER, GERB &amp; SOFFEN, LLP</b> <b>1180 Avenue of the Americas</b> <b>New York, NY 10036-8403</b>			
Tel: (212) 382 0700			
 SIGNATURE: <u>Edward A. Meilman</u> NAME <u>24,735</u> REGISTRATION NUMBER			

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of New York, New York  
Thomas RAUSCH Date: February 12, 2001  
Serial No.: Group Art Unit:  
Filed: Examiner:

For: TRANSGENIC PLANTS AND PLANT CELLS COMPRISING A REDUCED  
EXPRESSION OF INVERTASE INHIBITORS

Asst. Commissioner for Patents  
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to examination, please amend the application as follows:

IN THE CLAIMS:

Please amend claims 5-10, 12, 13, 15, 20 and 21 as follows.

Claim 5, line 1, change "any one of claims 1 to 4" to --claim 1--.

Claim 6, line 1, change "any one of claims 1 to 5" to --claim 1--.

Claim 7, line 1, change "any one of claims 1 to 6" to --claim 1--.

Claim 8, line 1, change "any one of claims 1 to 7" to --claim 1--.

Claim 9, line 1, change "any one of claims 1 to 8" to --claim 1--.

Claim 10, line 1, change "any one of claims 1 to 9" to --claim 1--.

Claim 12, line 1, change "any one of claims 1 to 11" to --claim 1--.

Claim 13, line 1, change "any one of claims 1 to 12" to --claim 1--.

Claim 15, line 1, delete "any one of";  
line 2, change "processes of claims 1 to 13" to --process of claim  
1--.

Claim 20, line 1, change "either one of claims 18 and 19" to --claim 18--.

Claim 21, line 1, change "any one of claims 18 to 20" to --claim 18--.

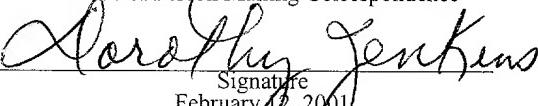
#### REMARKS

This Preliminary Amendment is submitted to change the multiple dependent claims to single dependent claims in order to eliminate the improper multiple dependent claims and to reduce the government filing fee.

#### EXPRESS MAIL CERTIFICATE

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Signature  
February 12, 2001  
Date of Signature

Respectfully submitted,



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51 PRTS

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## Transgenic Plants and Plant Cells With Reduced Expression of Invertase Inhibitors

### Description

This invention concerns transgenic plant cells and plants, a method for their

- 5 preparation and the use of invertase inhibitor cDNA sequences in an antisense or sense orientation to produce such plants.

Improvement in the quality and quantity of plant reserve material in seeds of dicotyledonous and monocotyledonous agriculturally-useful plants represents an important objective of biotechnology research. Hitherto, strategies generally were

- 10 developed which were based on the introduction of particular genes whose genetic products constituted enzymes which are themselves involved in the synthesis of the energy reserves (eg. ADP glucosepyrophosphorylase). Furthermore, methods are also described, in which an increased rate of glycolysis is obtained by modified expression of heterologous, and therefore, deregulated invertases or glucokinases
- 15 in the cytosol (DE-A1-195 29 696). In later variants the increased breakdown of sucrose by a deregulated fungal invertase, in combination with a deregulated bacterial glucokinase, leads to an increased rate of glycolysis. This approach rests on the assumption that the synthesis of stored oils in seeds is stimulated because of the increased concentrations of the intermediates of glycolysis, since the
- 20 metabolism of the primary photoassimilate, sucrose, is required for phosphorylated hexoses or the fatty acid precursors, pyruvate and acetyl coenzyme A.

- DE-A1-195 29 696 correspondingly describes the introduction of a foreign, eg. fungal gene for the expression of the invertase. Because of the supply of the
- 25 foreign gene, this fungal invertase enzyme (which is foreign and therefore is not subjected to any regulation) is formed in an amplified manner by regulating a suitable promoter, by means of which the decomposition of the sucrose catalysed by the invertase into glucose and fructose occurs faster. The resulting production of glucose at a higher rate is to bring about in the end an accelerated production of
- 30 plant reserve material. This process is based on an intervention in the metabolism in the cell of the seed storage tissue, whereby the assimilate transfer between maternal and seed parenchyma is affected only indirectly.

- The importance of cell wall invertase for the development of seeds high in starch and protein is well-known. Thus for example the starch accumulation in corn
- 35 seed is adversely affected with reduced expression of a cell wall invertase by interference in the assimilate transfer between pedicel and endosperm. Flower-

specific cell wall invertase isoforms for different plant species are well-known. For *Nicotiana tabacum*, it was able to be shown that an apoplastic invertase inhibitor is powerfully expressed, particularly in the ovary and stamens. Greiner *et al.* (Plant Physiol. (1998), 733-742) disclosed the amino acid sequence and cDNA sequence

5 of the mentioned invertase inhibitor as well as its in vitro demonstration of function by means of a heterologous expressed inhibitor protein. However, in vivo inhibition was still not indicated. Moreover, it is well-known that varying isoforms of cell wall invertases and invertase inhibitors exist in different tissues and at different times in plant development.

10 A specific classification of the activities and their possible combined effects of these two time-specific and tissue-specific occurring proteins has not been feasible until now. There are no known studies of an in vivo situation with regard to the regulation of cell wall invertases by invertase inhibitors. Just as little known, was if and when which isoforms of the cell wall invertases are subjected to  
 15 endogenous regulation by invertase inhibitors during the seed development and if so, which isoforms of the invertase inhibitors. That is why the specific use of these proteins for the production of beneficial plants has not been possible hitherto.

The technical problem of the invention therefore is to provide transgenic plant cells, plants and a method of producing these, in which the plants are  
 20 characterised by the production of seeds which, compared to seeds of untransformed plants, have a greater amount of plant reserve material such as carbohydrates, fats or proteins, without endogenous or exogenous proteins being over-expressed and without the phenotype of the plant and its development being impaired.

25 The technical problem underlying this invention is provided by a process for producing a transgenic plant with a deregulated invertase activity which stimulates plant development, whereby the process provides for: a nucleotide sequence of an invertase inhibitor to be produced from a cDNA bank of a cell suspension culture or from flowers with young ovules from a plant, or to be derived therefrom; a plant cell  
 30 of a plant of the same type or variety with a DNA construct, containing the functional nucleotide sequence of an invertase inhibitor bound to at least one regulatory unit to be transformed, cultivated and regenerated to a plant whose seed produces a greater amount of reserve material such as carbohydrates, fat or protein in comparison with plants not transformed with such a DNA construct.

35 The invention provides in particular for the production of transgenic plants with a modified expression of an invertase inhibitor, preferably an apoplastic

invertase inhibitor, whereby the plants are characterised by the expression of invertase inhibitor proteins being reduced or completely eliminated during seed development. The process can be applied advantageously to the most widely different dicotyledonous or monocotyledonous useful plants, for example: rape,

- 5 sunflower, peanut, oil palm, soy bean, *Calendula officinalis*, *Coriandrum sativum*, *Crambe abyssinica*, *Cuphea* ssp., *Dimorphotheca pluvialis*, *Euphorbia lagascae*, *Euphorbia lathyris*, *Lesquerella grandiflora*, *Limnanthes alba*, *Linum usitatissimum*, *Lunaria annua*, *Lunaria biennis*, *Oenothera* ssp., *Ricinus communis*, and *Simmondsia chinensis* as plants with seeds storing fat; corn, rice, wheat, barley,
- 10 oats, and rye as plants with seeds storing starch; and soy bean or pea, for instance, as plants with seeds storing protein.

Accordingly the invention provides for the transformation of a plant cell with a nucleotide sequence of an invertase inhibitor gene controlled by at least one regulatory unit, the nucleotide sequence of the invertase inhibitor gene being

- 15 capable of eliminating or reducing the activity of a cell-specific endogenous invertase inhibitor. In a preferred form of implementation, elimination of the activity of an endogenous invertase inhibitor in the cell can be achieved, in that the nucleotide sequence of an invertase inhibitor is inserted in an antisense DNA construct, ie. a construct in which a nucleotide sequence of the invertase inhibitor
- 20 gene is in an antisense orientation with respect to a promoter. Through the expression, ie. in this context the transcription of the antisense construct, the activity of the cell-specific invertase inhibitor gene is blocked or reduced so that the invertase deregulated in this way leads to an increased accumulation of reserve material in the seed.

25 In the context of this invention, an antisense construct is understood to mean a DNA construct which has a nucleotide sequence of an invertase inhibitor functionally bound in an antisense orientation to a promoter, this nucleotide sequence being either the full-length cDNA of the invertase inhibitor, a derived sequence thereof or a fragment, an allelic variant or derivative thereof.

- 30 In the context of this invention, a sequence derived from a cDNA is understood to mean a man-made or natural nucleotide sequence hybridising with this cDNA sequence, and therefore a nucleotide sequence hybridised with the cDNA sequence of the invertase inhibitor under the conditions described in Sambrook *et al.* (*Molecular Cloning, a laboratory manual*, 2<sup>nd</sup> edition (1989), Cold
- 35 Spring Harbor Laboratory Press), preferably under stringent conditions. According to the invention, hybridising sequences have a sequence identity of 60, 70, 80, 90,

95 or 97%, especially preferred 99%, of the cDNA sequence of an invertase inhibitor gene. Provided that fragments of a cDNA sequence of an invertase inhibitor are used according to the invention, the fragments have at least a length and sequence similarity, which is sufficient, due to hybridisation of a wild-type

- 5 transcript, to inhibit the translation of an endogenously produced invertase inhibitor mRNA, for example a length of a few hundred base pairs. Obviously provision can also be made for the antisense constructs to have nucleotide sequences of an invertase inhibitor gene or to consist of those which are transcribed but not translated, ie. untranslated regions, or so-called UTR's.

10 In the context of this invention, DNA constructs, which can cause the elimination or reduction of the activity of an endogenous invertase inhibitor gene, are understood to also mean DNA constructs which have a nucleotide sequence of an invertase inhibitor as defined above or a sequence derived therefrom, which are functionally bound in a sense orientation to at least one regulatory unit, eg. a

- 15 promoter. With constructs of this type, the production of endogenous invertase inhibitors can be prevented by co-suppression, for example by a plurality of sense copies of the nucleotide sequence of an invertase inhibitor being available in the genome of the transformed cell and eliminating the expression of endogenous invertase inhibitors.

20 The constructs according to the invention are preferably arranged in a vector, eg. in a plasmid, virus, cosmid, bacteriophage or one other standard vector in gene technology.

Provision can be made according to the invention to not only functionally bind the nucleotide sequence of an invertase inhibitor, which is to be used, to a 5'-wards

- 25 located promoter, but advantageously also to insert a transcription termination signal eg. from the NOS gene of *Agrobacterium tumefaciens* 3'-wards of the nucleotide sequence. Obviously it also possible to provide additional functional units in the vector, such as T-DNA border sequences or elements stabilising the vectors.

- 30 The invention in a preferred way therefore provides plants which have in their seeds an increased amount of reserve material in comparison with untransformed plants, whereby an amount of reserve material increased in comparison means that the average amount of reserve material studied in seeds of a totality of transformed plants is greater than the average amount of the reserve material in question in the
- 35 seeds of a totality of untransformed plants by predominantly 5, preferably 10, 20, 30, 40, 50, especially preferred 90, 100, 200 or 300%.

The invention therefore concerns the surprising science that, by means of a nucleotide sequence of an invertase inhibitor gene, in particular a cDNA of an invertase inhibitor gene, plants can be produced which *in vivo* have an increased accumulation of reserve material in the seed, without modifying or impairing the

- 5 development of the plant in other respects. As well, the invention is based *inter alia* on the surprising fact that the endogenous invertases are subject to an endogenous regulation by invertase inhibitors during the seed development.

The invention therefore also concerns the use of a nucleotide sequence of an invertase inhibitor gene functionally bound to at least one regulatory unit for the

- 10 transformation and production of plants which have a modified seed development, and in particular which produce seeds which have an increased amount of reserve material compared to seeds of untransformed plants.

The invention particularly concerns the afore-mentioned use of a nucleotide sequence of an invertase inhibitor gene, this having been obtained from a cDNA

- 15 bank of a cell suspension culture or from flowers with young ovules of the plant type or if necessary of the plant variety, or having been derived therefrom, which is to be transformed according to the invention with the DNA construct of this invention produced by means of this nucleotide sequence. The nucleotide sequence used for the transformation is consequently the nucleotide sequence or  
20 is derived therefrom, which encodes for the predominant isoform of the invertase inhibitor in the cell suspension culture or in the flowers with young ovules.

In the context of this invention, flowers with young ovules are flowers with immature ovules, that is after pollination but before the start of dormancy.

The solution to the technical problem according to the invention is also a

- 25 transgenic plant cell in which, in comparison with untransformed plant cells, an increased activity of the cell wall invertase is present because of reduced expression of invertase inhibitors, in which transgenic plant cell this reduced expression is produced in an antisense orientation by introducing an invertase inhibitor cDNA corresponding (homologous) to the same plant type and by  
30 regulating a promoter. According to the invention, a process for obtaining invertase inhibitor cDNA sequences in a sense orientation or antisense orientation is also provided, the process containing-independently of the respective species-the following steps:

- a) producing an inhibitor protein fraction from the cell wall protein fraction of an  
35 appropriate cell suspension culture

- b) production of corresponding peptide sequences after separation and purification of the peptide formed
- c) cloning firstly partial cDNA and subsequently full-length cDNA for the invertase inhibitor protein from a cDNA bank
- 5 d) cloning of the invertase inhibitor cDNA in a sense orientation or antisense orientation in a vector, eg. a binary vector
- e) transformation of the plant species with the sense orientation or antisense construct.

The assimilate transfer between maternal tissue and seed tissue is the rate-

- 10 determining step in the production of plant reserve material in seeds. If this step is accelerated by increasing the activity of the cell wall invertase expressed in the transfer zone, the result is an increased accumulation of the principal reserve material of the respective plant type (starch, fat, protein) as a result of the increased assimilate transfer (the increased activity of the cell wall invertase
- 15 causes an increase in the assimilate transfer between maternal and seed tissue).

The invention also concerns a process for producing the afore-mentioned plants, whereby in a first step an inhibitor protein fraction, in particular the predominant inhibitor protein fraction, from a cell wall protein fraction is obtained from a cell suspension culture or from flowers with young ovules; then in a second

- 20 step the inhibitor protein fraction is purified, if necessary separated, and at least N-terminal is sequenced, so that a nucleotide can be derived from the amino acid sequence thus obtained; within the framework of a third step by means of for example primers, partial or full-length cDNA for the invertase inhibitor protein is cloned from a cDNA bank of a cell suspension culture or from flowers with young
- 25 ovules of the same plant or variety mentioned above, then in a fourth step the cDNA obtained is cloned in a vector in a sense orientation or antisense orientation, in order to subsequently transform in a fifth step a plant cell of the same type or variety with the DNA construct thus obtained, this being the type or variety from which the cDNA and the amino acid sequence for the cDNA isolation was obtained.

- 30 From that, the invention also concerns plants, and plant components such as root, stem, leaves, cropping and propagating material such as fruit, pollen, seeds, husk, embryo, seedlings, cell cultures, callus tissues etc., produced in accordance with this process. The invention concerns as well any variety or type of plant, and accordingly has no specificity with regard to variety or type whatsoever. The
- 35 process according to the invention represents an essentially technical process, whereby within its framework a specific allocation of starting material for the means

to be used, such as cDNA sequences, is given to a plant ie. the target, which is to be transformed.

Unlike all previous methods, the invention described here is based therefore on the regulation of specific cell wall invertase isoforms expressed during the seed

- 5 development. The invertase inhibitor cDNA in a preferred form of implementation encodes for an apoplastic variant of the invertase inhibitor. By introducing a single autologous cDNA sequence of plant origin, ie. a cDNA sequence stemming from the organism to be transformed or derived therefrom, the decisive step in the separation of sucrose for the assimilate transfer is modulated at the natural location
- 10 of activity. The observation that the introduction of at least one sequence, that is of one invertase inhibitor cDNA, in a sense orientation or antisense orientation by controlling, for example, the constitutive CaMV35S promoter, the ubiquitin promoter or zein promoter from corn or a promoter of similarly high or greater activity, eg. a tissue-specific promoter as well which does not affect entire vegetative plant
- 15 development, but results in a specific deregulation only during the seed development, shows the extremely high specificity of the transgenic intervention. The advantages of this direct deregulation are obvious: 1) one single gene construct is sufficient to achieve a significant modification of reserve material accumulation; 2) no foreign gene products are produced; 3) the intervention in the
- 20 metabolism is extremely specific; 4) for tobacco, it is shown by example that the modified expression of an apoplastic invertase inhibitor leads to drastic changes in the production of stored oil.

- The modulation, particularly the increase in the accumulation of the seed reserve materials, by a specific modification of the expression of the invertase inhibitor, especially reduction, is based inter alia on the following mechanisms:
- 25 a) by modifying the activity phase of the cell wall invertase in the maternal tissue, the efficiency of the nutrient load is affected, ie. is increased for example in inhibitor antisense transforms.
  - b) The oxidative pentose phosphate cycle is of crucial significance for the synthesis
  - 30 of reserve oils of the seeds. The sustained increased availability of glucose in, for example, inhibitor antisense transforms therefore promotes the synthesis of stored oil.
  - c) by modifying the ratio of hexoses to sucrose, the cell division phase of the seed development is affected. By extending the activity phase of the cell wall invertase
  - 35 in, for example, inhibitor antisense transforms, the cell count per seed, for instance, is increased.

In comparison with the equally possible overexpression of the cell wall invertase(s) involved in the assimilate transfer, the approach of turning off the invertase inhibitors described here indirectly has even further advantages. The cell wall invertases expressed during seed production are heavily expressed naturally,

- 5 the scope of additional induction by using strong promoters is limited by this, whereas a large increase in the activity of the cell wall invertase(s) can be obtained by the antisense switching off according to the invention of the inhibitor. In fact, by expressing a heterologous, deregulated, inhibitor-sensitive invertase with a signal peptide for the target control in the cell wall space, a similar effect could be
- 10 achieved, but in this case foreign proteins have to be used. In addition, with a combination of the seed-specific promoters with a deregulated invertase used for this approach, there is a high risk that too great an expression results in undesired side effects. In contrast to this, in the process described here, the maximum activity of the naturally-occurring cell wall invertases is never exceeded, merely the time
- 15 span of its activity is prolonged during the accumulation of reserve material. For these reasons, in each case of introducing a heterologous invertase, indirect regulation of the cell wall invertases is to be preferred over antisense expression of invertase inhibitors or, in the framework of the co-suppression technology, over sense DNA constructs and represents a significant technical improvement.

20 **Methods for obtaining a homogenous inhibitor protein fraction from the apoplastic cell wall protein fraction of a cell suspension culture**

A cell suspension culture is started from the respective plant species. The process for obtaining a cell culture follows standard protocols of plant tissue culture. As a rule, the cells are started as a shaking culture under sterile conditions

- 25 in a complex nutrient medium with the addition of sucrose (carbon source). Under these cultivation conditions, plant cells express a cell wall invertase which is regulated by a likewise expressed invertase inhibitor.

The accumulation and purification of the invertase inhibitor is based on its binding to the cell wall invertase. First a cell wall protein fraction is extracted

- 30 through incubation in 1M NaCl, 1mM PMSF at 4°C while shaking. Usually no cytosolic proteins are extracted doing this. The cell wall protein fraction obtained in this way is concentrated through ammonium sulfate precipitation (80%) or through membrane filtration. By means of subsequent chromatography in a concanavalin A column, a glycoprotein fraction is obtained which contains the glycosylated cell wall
- 35 invertase and the invertase inhibitor bound to this. SDS-PAGE/Western blot analyses of the cell wall invertase fractions, and therefore invertase inhibitor-

enriched fractions, obtained in this way with a polyclonal antiserum against the invertase inhibitor from tobacco cells, indicate the presence of invertase inhibitors, generally proteins of 15-25kDa.

Figure 1 shows the detection of invertase inhibitors of other plant types which

- 5 are homologous to the tobacco invertase inhibitor-a Western blot analysis of cell wall protein samples obtained from suspension cultures of *Chenopodium rubrum* (1) and *Daucus carota* (2). The development was carried out with an antiserum produced against the recombinant tobacco invertase inhibitor. Invertase inhibitor polypeptides of approx. 17kDa were detected for both species.

- 10 Further purification of the complexes consisting of cell wall invertase and invertase inhibitor is carried out through ion-exchange chromatography in a cation-exchanger, eg. sulfopropylsephadex. After sequential chromatography, first of all via a pH gradient (pH8-12), after that via an NaCl gradient, a highly enriched preparation of the cell wall invertase is obtained in which the invertase inhibitor is
- 15 present with the latter in the stable complex. The peak fractions of the final ion exchange purification are detected through SDS-PAGE/Western blot analysis because of cell wall invertase eg. with an antiserum against the carrot cell wall invertases. Moreover, the invertase activities of all fractions were determined in the coupled enzymatic test with hexokinase/glucose-6-phosphate dehydrogenase. The
- 20 fractions with the strong cell wall invertase immune signal but low invertase activity contain the generally high-purity inhibitor protein.

#### **Methods for obtaining peptide sequences of the purified invertase inhibitors**

- After the purification protocol described above, the inhibitor protein is sufficiently pure to become N-terminal unsequenced after electroblotting directly onto a PMDF membrane. After obtaining 100-500µg of inhibitor protein, this can if necessary be purified again via SDS-PAGE and then can be digested by trypsin directly in the gel. The separation of the resulting peptides through reverse phase HPLC and its subsequent sequencing through Edman degradation is in accordance with standard procedures. The combination of N-terminal
- 25 unsequencing and the sequencing of the peptides preserved during tryptic digestion results in sufficient sequencing information for cloning based on the RT-PCR process

#### **Process for cloning, firstly, partial cDNA and subsequently full-length cDNA for the respective invertase inhibitor protein from a cDNA bank**

- 35 Starting from the preserved peptide sequence information, primer sequences are derived according to the genetic code. Standard algorithms are used for the optimum primer design. In another implementation, primers are designed with

highly conservative sequence regions of the already known invertase inhibitor sequences from *Nicotiana tabacum*, *Lycopersicon esculentum*, *Arabidopsis thaliana* and *Citrus inshui*.

First a single strand cDNA synthesis is carried out according to standard

- 5 procedures. For this, complete RNA is extracted in accordance with standard procedures from a cell suspension culture, or, in another implementation, from flowers with young ovules. A partial invertase inhibitor cDNA is then first amplified through RT-PCR. In one implementation, the amplification is cloned into the Bluescript vector. After sequencing and confirming a sequence homologous to the
- 10 known invertase inhibitors, this partial cDNA is used as a probe for the production of full-length clones. For this, either a cDNA bank from a cell suspension culture, or in another implementation, a cDNA bank from flowers with young ovules, is started in accordance with standard procedures.

**15 Cloning of the invertase inhibitor cDNA in a sense orientation or an antisense orientation in a vector, for example a binary vector**

- In one form of implementation the invertase inhibitor cDNA cloned for each plant type is cloned in the binary vector BinAR (Bin19 derivative) (Höfgen and Willmitzer, Plant Sci. 66 (1990), 221-230). In one form of implementation, the CaMV35S promoter is used for both for antisense and sense constructs, but if necessary other promoters, for example tissue-specific promoters, also can be used.

Figure 2 shows the binary vector (BinAR) for the *Agrobacterium tumefaciens*-mediated transformation. The encoding regions of the invertase inhibitor cDNA are cloned in a sense orientation or antisense orientation in the “multiple cloning site”.

**25 Transformation of the plant species with the sense and antisense gene constructs respectively**

- For most dicotyledonous useful plants, invertase inhibitor sense/antisense transforms are obtained by using *Agrobacterium tumefaciens*-mediated transformation (standard process). Accordingly an *Agrobacterium* is used, since it
- 30 contains recombinant DNA molecules which have invertase inhibitor cDNA in antisense orientation or sense orientation, ie. in which the invertase inhibitor cDNA exists 3'-wards from a promoter and functionally bound with the latter. In a form of implementation useable for many plant types, leaf fragments are transformed with this, primary transforms being regenerated from the recombinant cells in an
- 35 antibiotic-containing medium. The transformation technique actually selected is dependent on the plant type. A transgenic plant is obtained by regeneration of a transformed plant cell.

The influence of the modified expression of an apoplastic invertase inhibitor in *Nicotiana tabacum* is described below:

Tobacco (*Nicotiana tabacum*) was transformed in sense orientation and antisense orientation with the cDNA of the apoplastic tobacco invertase inhibitor

- 5 (clone Nt-inhl; Greiner et al., loc cit. 1998) (*Agrobacterium tumefaciens*-mediated transformation, BinAR vector, CaMV35S promoter; leaf slice transformation according to standard procedures). The cDNA used was obtained from a cell suspension culture of tobacco. Primary transforms were first regenerated to plants via tissue culture and subsequently brought to flower in the greenhouse. The seeds
- 10 of the primary transforms were sown on a kanamycin-containing medium. After sterile pre-cultivation the plants of the F1 generation were brought to flower in the greenhouse. After pollination the cell wall invertase activities in the ovaries were determined at regular time intervals.

Figure 3 shows cell wall invertase activities in the ovary of a tobacco wild-type

- 15 (SNN), a representative inhibitor antisense transform (as16) and a representative inhibitor sense transform (s9) during the early seed development (0-14 days after fertilisation). The activities are indicated in mmol glucose/g fresh weight/min.

The amount of invertase inhibitor protein was determined through Western blot analysis.

- 20 With regard to this, Figure 4 shows the proof of the selective reduction (antisense transform) or increase (sense transform) of the invertase inhibitor polypeptide in stamens and ovaries, detected with an antiserum directed against the recombinant tobacco invertase inhibitor (sense transform (s9): 1, ovaries; 2, stamens. Antisense transform: 3, ovaries; 4, stamens. Wild-type (SNN): 5, ovaries; 25 6, stamens). Purified samples, which contain an inhibitor bound only to a cell wall invertase, were plotted through concanavalin A chromatography.

After maturation of the seeds, their dry weights were determined, as well as the amount of stored oil and total protein.

Measurement of the cell wall invertase activities during the early seed

- 30 development (Fig. 3) shows first of all an approximately 6-fold increase for the wild-type between the 6<sup>th</sup> and 12<sup>th</sup> day after pollination. This period corresponds to the late cell division phase and the beginning of the storage phase. The Western blot analysis shows that in the ovary the amount of invertase inhibitor polypeptide in antisense transforms is sharply decreased, however it is greatly increased in
- 35 antisense transforms (Fig. 4). In contrast to this, the modified inhibitor expression is

obtained only slightly in stamens, presumably because several inhibitor isoforms are expressed in this tissue.

The modified activity of the cell wall invertase during the seed development has an effect on the dry weight/seed (Table 1), the stored oil content/seed (Table 5 2) and the total protein content/seed (Table 3), but not on the total number of seeds per flower and also not on the seed size.

**Table 1**

Dry weight per seed in tobacco wild-type (SNN), in two representative inhibitor sense transforms (s9, s10) and in two representative inhibitor antisense transforms (as16, as43).

Tobacco Line	µg Dry Weight/Seed	Percent of Wild-type (SNN)
WT (SNN)	64 ± 2	100
s9	42 ± 2	66
s10	52 ± 2	81
as16	71 ± 1	110
as43	86 ± 4	134

**Table 2**

Seed oil content per seed in tobacco wild-type (SNN), in two representative inhibitor sense transforms (s9, s10) and in two representative inhibitor antisense transforms (as16, as43).

Tobacco Line	µg Total Oil/Seed	Percent of Wild-type (SNN)
WT (SNN)	23	100
s9	10	43
s10	16	69
as16	28	122
as43	39	170

**Table 3**

Total protein per seed in tobacco wild-type (SNN), in two representative inhibitor sense transforms (s9, s10) and in two representative inhibitor antisense transforms (as16, as43).

Tobacco Line	µg Total Protein/Seed	Percent of Wild-type (SNN)
WT (SNN)	7.4	100
s9	5.5	74
s10	5.7	77
as16	7.8	105
as43	9.9	134

The large increase in stored oil content in two individual antisense transforms (+ 22% and + 70% respectively) correlates with increases in total protein and seed weight, the increase for stored oil being most marked. Remarkably the increase in 40 the cell wall invertase activity in the ovary during the seed development corresponds to the phase of the maximum accumulation of stored oil.

The entire vegetative development phase of the inhibitor antisense and inhibitor sense transforms proceeds with no visible phenotype, with the exception

of the germination process. Here there is no difference between the germination of tobacco wild-type seeds and invertase inhibitor antisense seeds, whereas with seeds of invertase inhibitor sense transforms there is a significant delay in germination.

- 5      Figure 5 shows the germination behaviour of seeds of tobacco wild-type (SNN), of invertase inhibitor antisense transforms (INHas) and of invertase inhibitor sense transforms. Per line, in each case 40 seeds were sown on an LS medium (0.5% sucrose, pH5.6) under sterile conditions. The protrusion of the radicle is used as the criterion for germination.

10     A further example of application of this invention is the introduction of an invertase inhibitor antisense construct in rape (*Brassica napus*). On a seed basis, rape contains an amount of stored oil comparable to tobacco. The result of an invertase inhibitor antisense transformation is an increase in the stored oil content by at least 20%, and possibly by up to 70%.

- 15     A further application with the same objective, namely increasing the stored oil content, is the transformation of the sunflower, or perhaps the transformation of the soy bean, with an invertase inhibitor antisense construct (making available transgenic oil-storing plants of these species).

In an additional form of implementation, the amount of seed stored starch is increased by putting invertase inhibitor antisense constructs into corn, rice, wheat, oats, barley and rye. Thus transgenic plant cells or plants, which store starch, can be provided. In a further form of implementation, for protein-rich seeds, eg. soy bean and pea, the total amount of stored protein is increased by introducing invertase inhibitor antisense constructs.

- 25     In an additional implementation form, the germinating capability of seeds of a useful plant is increased by introducing invertase inhibitor antisense constructs, or that is to say, by the enhanced reserve material accumulation resulting therefrom.

The preparation of transgenic plant cells or plants preferably concerns useful plants.

30

**New Claims 1 to 21**

1. A process for producing a transgenic plant having a deregulated invertase activity which stimulates plant development, whereby a nucleotide sequence of an invertase inhibitor gene is obtained from a cDNA bank of a cell suspension culture or from flowers with young ovules of a plant; whereby a plant cell of a plant of the same type or variety, from which the nucleotide sequence comes or is derived, is transformed with a DNA construct containing the functionally bound nucleotide sequence of an invertase inhibitor gene with at least one regulatory unit, is cultivated and a plant regenerated, whose seeds have an increased amount of reserve material in comparison with untransformed wild-type plants.
2. A process according to claim 1, in which the regulatory unit is a promoter.
3. A process according to claim 2, in which the promoter is a constitutive or inducible promoter.
4. A process according to claim 3, in which the promoter is the CaMV35S promoter or the ubiquitin promoter or zein promoter from corn.
5. A process according to any one of claims 1 to 4, in which the nucleotide sequence of an invertase inhibitor gene is a cDNA, a nucleotide sequence hybridising with that, or a fragment of one of the two.
6. A process according to any one of claims 1 to 5, in which the invertase inhibitor is an apoplastic invertase inhibitor.
7. A process according to any one of claims 1 to 6, in which the nucleotide sequence of an invertase inhibitor gene has a sense orientation or antisense orientation to the promoter.
8. A process according to any one of claims 1 to 7, in which the DNA construct has additional regulatory units, in particular a transcription termination signal.
9. A process according to any one of claims 1 to 8, in which the transcription termination signal comes from the NOS gene of *Agrobacterium tumefaciens*.
10. A process according to any one of claims 1 to 9, in which the plant cell is a cell of a dicotyledonous or monocotyledonous plant.
11. A process according to claim 10, in which the plant cell is a cell from rape, sunflower, peanut, soy bean, oil palm, rice, corn, wheat, barley, oats, rye, pea, *Calendula officinalis*, *Coriandrum sativum*, *Crambe abyssinica*, *Cuphea* ssp., *Dimorphotheca pluvialis*, *Euphorbia lagascae*, *Euphorbia lathyris*, *Lesquerella grandiflora*, *Limnanthes alba*, *Linum usitatissimum*, *Lunaria annua*, *Lunaria biennis*, *Oenothera* ssp., *Ricinus communis* or *Simmondsia chinensis*.

12. A process according to any one of claims 1 to 11, in which the DNA construct is in a vector, in particular a plasmid or virus.

13. A process according to any one of claims 1 to 12, in which the transformation of the plant cell is carried out by means of *Agrobacterium tumefaciens*,

5 a biolytic process, by means of electrically or chemically induced DNA absorption, electroporation, macroinjection, microinjection or PEG-mediated transformation.

14. A process for producing sequences containing invertase inhibitor cDNA in a sense orientation or antisense orientation in a vector, and comprising the following steps:

10 a) producing an inhibitor protein fraction from the cell wall protein fraction of a cell suspension culture or from flowers with young ovules,

b) production of corresponding peptide sequences after separation and purification of the peptide formed,

c) cloning firstly partial cDNA and subsequently full-length cDNA for the invertase

15 inhibitor protein from a cDNA bank, in particular from flowers with young ovules and

d) cloning of the invertase inhibitor cDNA in a sense orientation or antisense orientation in a vector, particularly a binary vector.

15. A transgenic plant which can be produced according to any one of the processes of claims 1 to 13, as well as a part thereof.

20 16. Propagating and cropping material of a plant according to claim 15.

17. Propagating and cropping material of a transgenic plant according to Claim 16, which propagating and cropping material is fruit, seed, seed husk, embryo, seedling, callus tissue, cell culture, stem, leaf or root.

18. Application of a functional nucleotide sequence of an invertase inhibitor  
25 gene bound to at least one regulatory unit for the transformation and production of transgenic plants, which have a modified seed development as a result of the transformation.

19. An application according to claim 18, whereby the modified seed development is a seed development in accordance with which the seeds of the  
30 transgenic plant have an increased amount of reserve material in comparison with seeds of an untransformed wild-type plant.

20. An application according to either one of claims 18 and 19, whereby the nucleotide sequence comes from, or is derived from, a cDNA bank from flowers with young ovules of the plant type or variety to be transformed.

21. An application according to any one of claims 18 to 20, whereby the nucleotide sequence is in an antisense orientation to at the very least one regulatory unit, in particular the promoter.

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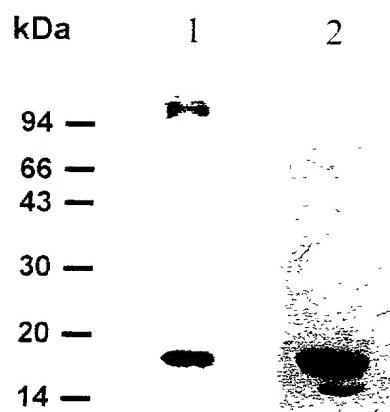


Fig. 1

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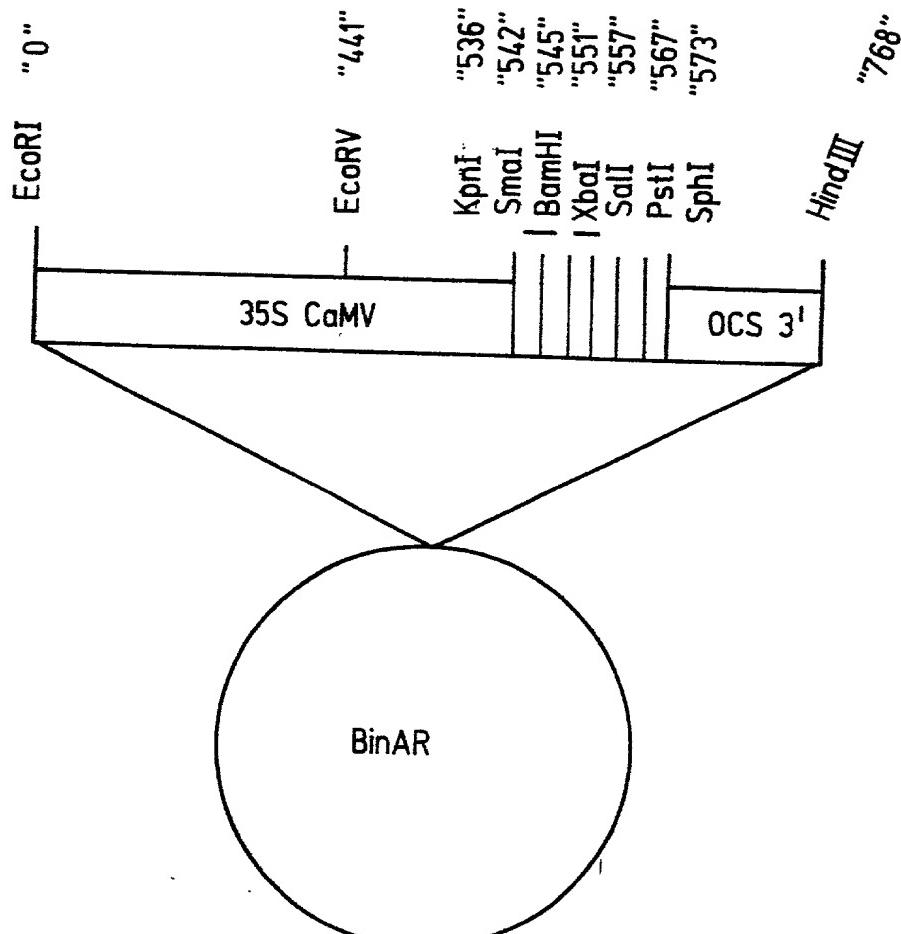


Fig.2

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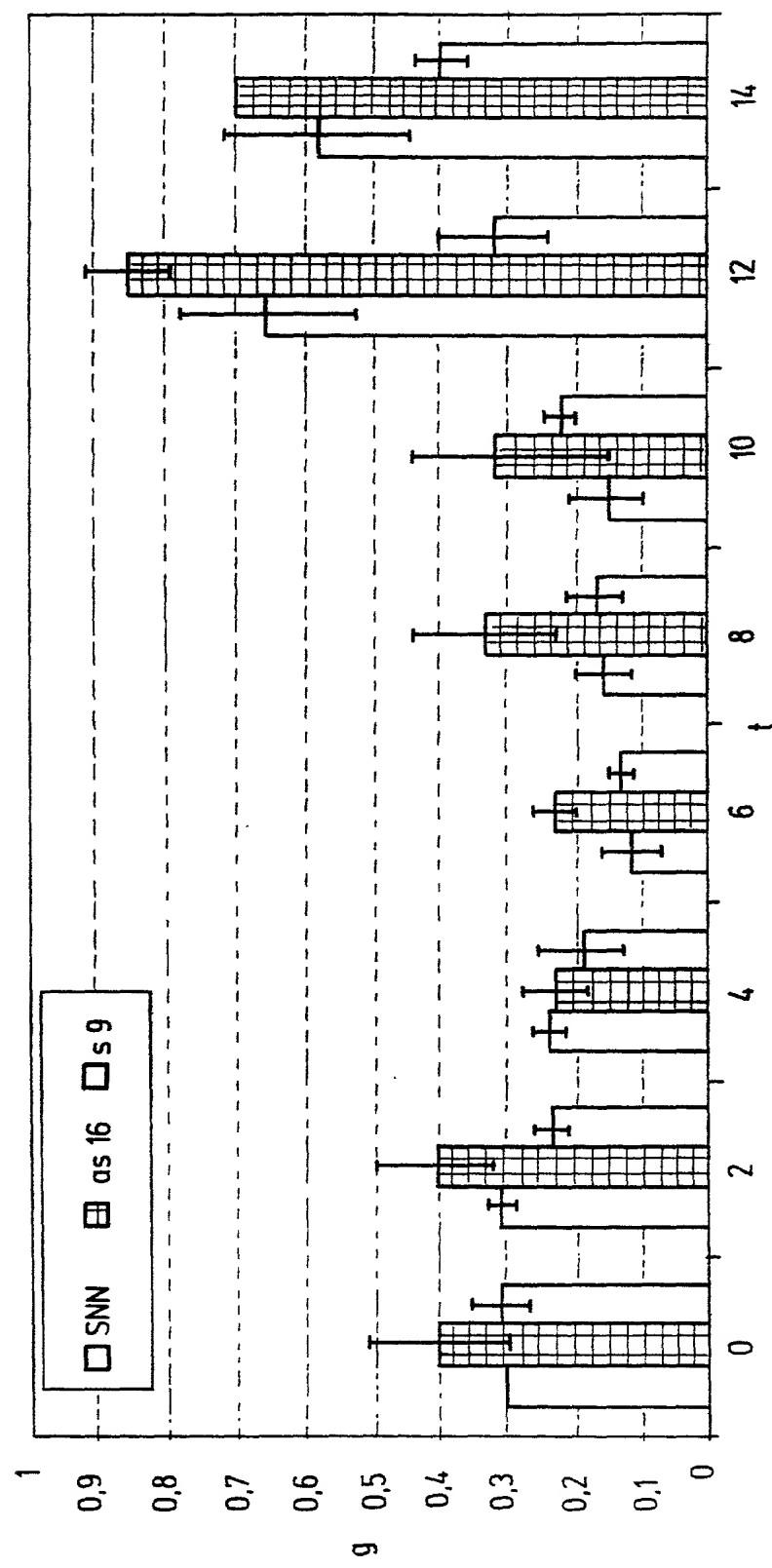


Fig.3

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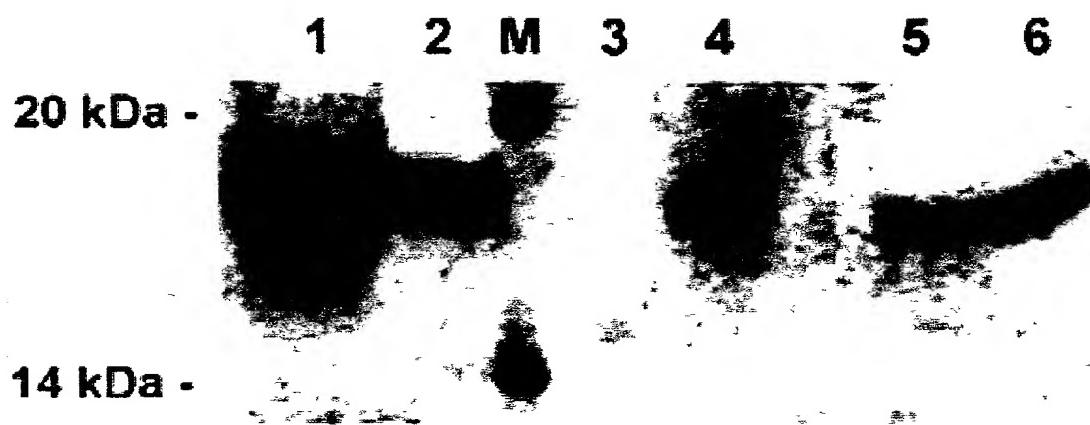


Fig. 4

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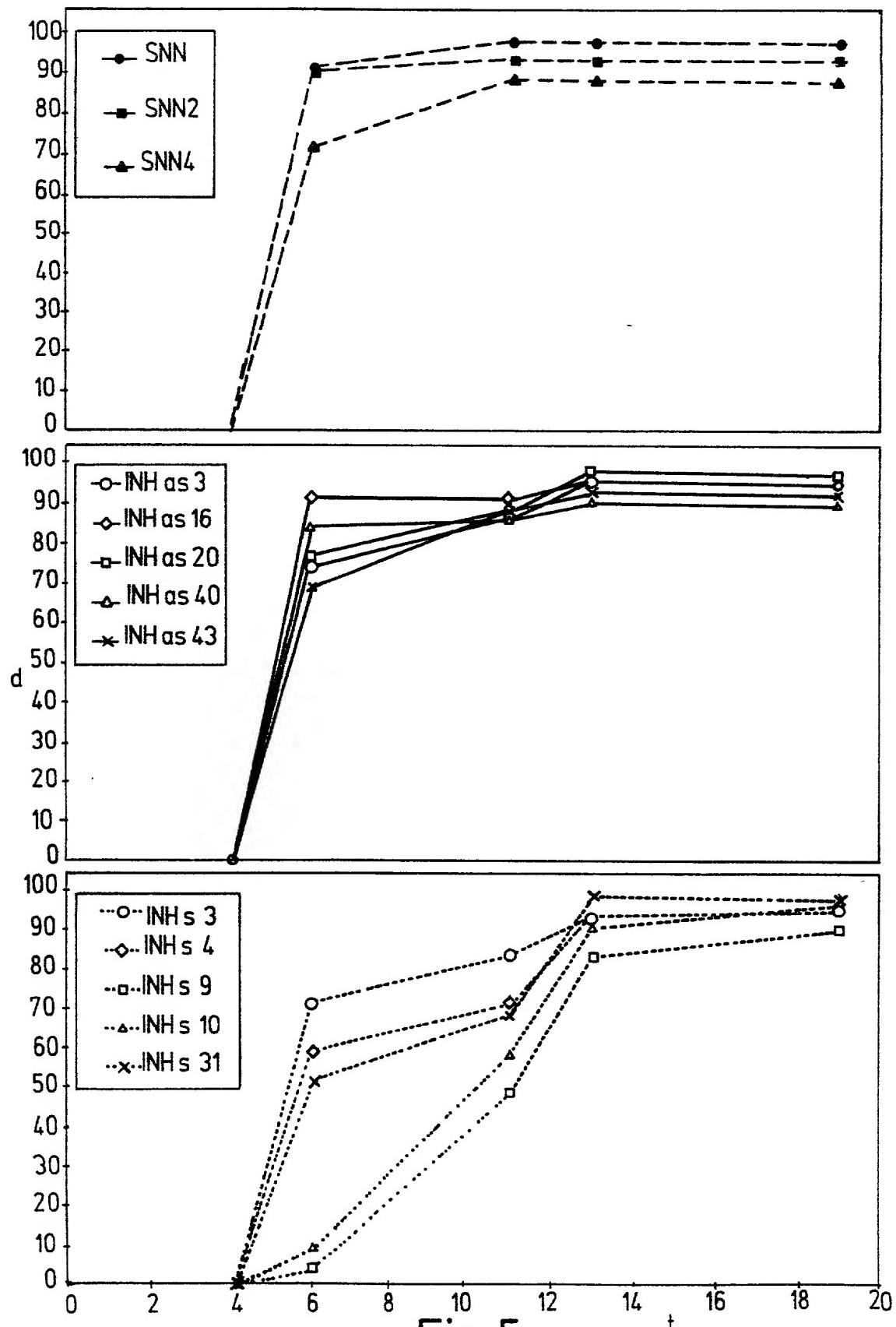


Fig. 5

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## UNITED STATES OF AMERICA

## COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

OFGS FILE NO.

P/2107-162

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TRANSGENIC PLANTS AND PLANT CELLS WITH REDUCED EXPRESSION OF  
INVERTASE INHIBITORS

the specification of which is attached hereto, unless the following box is checked:

was filed on 11 August 1999 as United States patent Application Number or PCT International patent

application number PCT/EP99/05890 and was amended on 6 September 2000 (if any).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim priority benefits under Title 35, United States Code §119 of any foreign application(s) for patent or inventor's certificate or United States provisional application(s) listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

## Prior Foreign or Provisional Application(s)

COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119
Germany	198 36 405.9 ✓	12 August 1998✓	YES <input checked="" type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

UNITED STATES APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)

I hereby appoint customer no. 2352 OSTROLENK, FABER, GERB & SOFFEN, LLP, and the members of the firm, Samuel H. Weiner - Reg. No. 18,510; Jerome M. Berliner - Reg. No. 18,653; Robert C. Faber - Reg. No. 24,322; Edward A. Meilman - Reg. No. 24,735; Stanley H. Lieberstein - Reg. No. 22,400; Steven L. Weisburd - Reg. No. 27,409; Max Moskowitz - Reg. No. 30,576; Stephen A. Soffen - Reg. No. 31,063; James A. Finder - Reg. No. 30,173; William O. Gray, III - Reg. No. 30,944; Louis C. Dujimich - Reg. No. 30,625; Douglas A. Miro - Reg. No. 31,643, and Michael J. Scheer - Reg. No. 34,425, as attorneys with full power of substitution and revocation to prosecute this application, to transact all business in the Patent & Trademark Office connected therewith and to receive all correspondence.

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FULL NAME OF SOLE OR FIRST INVENTOR <u>Thomas RAUSCH</u>	INVENTOR'S SIGNATURE	DATE <u>23.2.2001</u>
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FULL NAME OF SECOND JOINT INVENTOR (IF ANY)	INVENTOR'S SIGNATURE	DATE
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